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# Effects of Carbon Sources on Chemical Composition of Cell Envelopes of *Pseudomonas aeruginosa* in Association with Polymyxin Resistance

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Cells of *Pseudomonas aeruginosa* 015 were grown in basal medium with isobutyrate, DL-2-methylbutyrate, isovalerate, L-valine, L-isoleucine, L-leucine, D-glucose, or L-glutamate as the carbon source. Their resultant susceptibility to polymyxin B varied from a minimal inhibitory concentration of 2 U of polymyxin per ml for isobutyrate-grown cells to 975 U/ml for L-glutamate-grown cells. Cell envelopes from cells grown with each carbon source were compared with cell envelopes from cells grown in Mueller-Hinton broth as to their content of total protein, carbohydrate, and 2-keto-3-deoxyoctonate and as to their protein composition as determined by slab polyacrylamide gel electrophoresis. No pattern of cell envelope content of total protein, carbohydrate, 2-keto-3-deoxyoctonate, or outer membrane protein concentrations could be correlated with the degree of resistance to polymyxin. In these cells increased resistance to polymyxin was not associated with the loss of outer membrane proteins and lipopolysaccharide by the cell envelope.

Conrad et al. (4) recently demonstrated that growth of *Pseudomonas aeruginosa* in media with various branched-chain amino acids, their acyl derivatives, D-glucose, or L-glutamate as the carbon source affected the susceptibility of the cells to the antibiotics polymyxin B and colistin. The fluctuation in minimal inhibitory concentration (MIC) for polymyxin B ranged from 0.5 μg/ml for cells grown with either isobutyrate or DL-2-methylbutyrate as the carbon source to 375 μg/ml for cells grown on L-glutamate (4).

The polymyxin antibiotics exert their antimicrobial action on gram-negative bacteria by disrupting the osmotic equilibrium of the cell and causing leakage of the cell contents (21, 24, 29) as a result of the interaction between the antibiotic and the phospholipids in the cytoplasmic membrane (1, 27, 30). To gain entry to the cytoplasmic membrane, the antibiotic molecule initially binds to lipopolysaccharide (LPS) and phospholipids in the outer membrane (OM) (1, 20, 22, 27, 30, 31) and then penetrates through the cell wall layers in a manner that has yet to be determined. Ultrastructural studies of *P. aeruginosa* treated with polymyxin have supported this mechanism of action (11, 16).

Resistance to polymyxin in *P. aeruginosa* appears to be due to an exclusion mechanism (1, 9, 11) which acts to prevent the antibiotic from penetrating through the OM to reach the sensitive sites on the cytoplasmic membrane. This loss of permeability to the antibiotic could be gained by altering the OM in one of two ways. One possibility would be that the loss of one or more of the major OM proteins which might act as a porin to facilitate the penetration of polymyxin through the OM barrier would render the OM impermeable to the antibiotic. Evidence to support the loss of OM proteins as a basis of resistance to polymyxin B in *P. aeruginosa* has been given recently by Gilleland and Lyle (10). The acquisition of polymyxin resistance in the strains employed in their study was associated with a loss of three OM proteins having apparent molecular weights of 24,000, 36,500, and 47,000. In addition, the LPS content of these strains was reduced approximately 25%. Another possible way to gain resistance would be the alteration of the lipid composition of the OM in such a way that polymyxin no longer could bind to and penetrate the OM. Conrad et al. (4) have shown previously that lipid alterations are associated with changes in polymyxin susceptibility when *P. aeruginosa* is grown with various carbon sources. However, in their study these authors did not assess protein alterations.

The purpose of the present study was to determine whether the cell envelopes of *P. aeruginosa* cells which had varying resistance to polymyxin as a result of variation in the carbon source (4) were altered in their composition of OM proteins and LPS in a manner similar to

that reported by Gilleland and Lyle (10). No association between increased polymyxin resistance and alterations in the cell envelope content of LPS and OM proteins was observed.

## MATERIALS AND METHODS

**Organism.** *P. aeruginosa* strain 015, a clinical isolate furnished by University Hospital, Oklahoma City, Okla., and previously employed in the Conrad et al. study (4), was used. Stock cultures were maintained on Mueller-Hinton slants and transferred every 3 weeks.

**Growth conditions.** The cells were grown using Jacobson medium (15) as the base medium. This defined medium, in which the final concentrations of trace elements and nitrogen source were not growth limiting, contained the following: 37 mM potassium phosphate buffer (pH 6.8); 40 mM $NH_4Cl$; 1.6 mM $MgCl_2$; 0.3 mM $MnCl_2$; trace amounts of $Fe^{2+}$, $Ca^{2+}$, NaCl, and $NaMoO_4$; and a carbon source at 25 mM concentration. The following compounds were used individually as carbon sources: isobutyrate, DL-2-methylbutyrate, isovalerate, L-valine, L-isoleucine, L-leucine, D-glucose, and L-glutamate. The acidic acyl derivatives were neutralized with 1 M KOH to pH 7.0 before addition to the medium. Cells grown in Mueller-Hinton broth (Difco Laboratories) were used as control cells. All cells were harvested for the production of cell envelopes after they had reached the late logarithmic growth phase. All growth was accomplished at 37°C with aeration provided by vigorous shaking in a Lab-Line Environ-Shaker 3597-1 shaker employing 2-liter Erlenmeyer flasks containing 1 liter of medium.

**Determination of MIC.** The minimal inhibitory concentration (MIC) of polymyxin against the cells grown with the various carbon sources was measured by a modification of the method of Washington and Barry (32) as described previously by Conrad et al. (4). The MIC obtained for each carbon source was determined after 48 h of incubation. All MICs were the mean of four to six separate determinations.

**Cell envelope production.** Cell envelopes were prepared by the procedure of Stinnett et al. (26), using cells harvested from 6 liters of medium.

**Chemical analyses.** Total protein was estimated by the Hartree modification (14) of the Lowry method with bovine serum albumin as the standard. Carbohydrate was determined by the anthrone method (23) with glucose as the standard. 2-Keto-3-deoxyoctonate (KDO) was estimated in samples after hydrolysis at 100°C for 8 min in 0.25 N $H_2SO_4$ by the method of Dröge et al. (7). Authentic KDO (Sigma Chemical Co., St. Louis, Mo.) was used as the standard.

**Readily extractable lipid.** The protocol for obtaining the readily extractable lipid fraction has been previously described (4). The cellular percentage of readily extractable lipid was measured gravimetrically by evaporating 10 ml of the extract to dryness at room temperature in tared aluminum weigh pans.

**Statistics.** The statistical significance of the values obtained in the chemical analyses was determined by performing Student's *t* test on the means, employing the one-tailed *t* table (3).

**Electrophoresis procedures.** For polyacrylamide gel electrophoresis, proteins were solubilized from the cell envelopes by heating at 100°C for 2 min in sample buffer comprised of 0.0312 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8) with 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 10% urea. Slab gel electrophoresis was performed with a Studier gel apparatus (model 220, Bio-Rad Laboratories, Richmond, Calif.). The discontinuous sodium dodecyl sulfate method employed was the modification by Lugtenberg et al. (18) of the Laemmli procedure (17), utilizing gels of 15% acrylamide. A 125-μg sample of cell envelope, which contained approximately 60 to 65 μg of protein, was added to each slot of the gel apparatus in a volume of 100 μl. The following proteins (with corresponding molecular weights) were used as standards: albumin, 68,000; ovalbumin, 43,000; lactic dehydrogenase, 36,000; and trypsin, 23,000. Electrophoresis was performed at room temperature, using a constant current of 30 mA. Gels were stained by the four-step method of Fairbanks et al. (8).

**Molecular weight calculations.** The apparent molecular weights of the protein bands of interest were calculated by the method of Weber and Osborn (33), using relative electrophoretic mobilities.

## RESULTS

**MICs.** The carbon source provided in the growth medium significantly influenced the degree of resistance to polymyxin B in this strain of *P. aeruginosa*. As shown in Table 1, growth in medium having as the carbon source isobutyrate, DL-2-methylbutyrate, isovalerate, L-valine, or L-isoleucine rendered the cells more susceptible to polymyxin B than were control cells grown in Mueller-Hinton broth. After growth with L-leucine, D-glucose, or L-glutamate as the carbon source, the cells became more resistant to polymyxin B than were the control cells. The observed range of susceptibility to polymyxin varied significantly, with cells grown in L-glutamate being nearly 500 times more resistant to the antibiotic than cells grown in isobutyrate.

**Chemical analyses.** No discernible pattern of the cell envelope content of total protein, carbohydrate, or KDO could be associated with

TABLE 1. *Relationship between carbon source and susceptibility to polymyxin B in P. aeruginosa 015*

| Carbon source | MIC (U/ml) of polymyxin B |
|---|---|
| Mueller-Hinton medium | 85 |
| Isobutyrate | 2 |
| DL-2-Methylbutyrate | 4 |
| Isovalerate | 16 |
| L-Valine | 16 |
| L-Isoleucine | 48 |
| L-Leucine | 170 |
| D-Glucose | 650 |
| L-Glutamate | 975 |

TABLE 2. *Chemical analyses of cell envelopes of P. aeruginosa 015 grown on various carbon sources*[a]

| CARBON SOURCE | TOTAL PROTEIN | | | CARBOHYDRATE | | | KDO | | |
|---|---|---|---|---|---|---|---|---|---|
| | MEAN[b] | PERCENT[c] CHANGE | P VALUE[d] | MEAN | PERCENT CHANGE | P VALUE | MEAN | PERCENT CHANGE | P VALUE |
| Mueller-Hinton | 51.83 (±2.5) | – | – | 3.86 (±0.40) | – | – | 0.90 (±0.07) | – | – |
| L-Leucine | 54.33 (±2.5) | +4.82 | > .05 | 4.52 (±0.50) | +17.10 | < .05 | 0.78 (±0.07) | -13.33 | < .025 |
| D-Glucose | 56.83 (±4.2) | +9.65 | < .05 | 3.74 (±0.40) | -3.10 | > .30 | 0.65 (±0.02) | -27.78 | < .0005 |
| L-Glutamate | 51.83 (±2.5) | 0.00 | – | 3.27 (±0.25) | -15.28 | < .025 | 0.88 (±0.05) | -2.22 | > .30 |
| Isobutyrate | 56.50 (±5.4) | +9.01 | > .05 | 3.27 (±0.38) | -15.28 | < .05 | 0.69 (±0.10) | -23.33 | < .05 |
| DL-2-Methylbutyrate | 56.17 (±3.3) | +8.37 | < .025 | 3.67 (±0.17) | -4.92 | > .20 | 0.73 (±0.07) | -18.89 | < .005 |
| Isovalerate | 52.00 (±2.5) | +0.33 | > .30 | 4.30 (±0.25) | +11.40 | > .05 | 0.79 (±0.07) | -12.22 | < .025 |
| L-Valine | 56.00 (±5.4) | +8.05 | > .05 | 4.76 (±0.17) | +23.32 | < .005 | 0.80 (±0.07) | -11.11 | < .05 |
| L-Isoleucine | 52.67 (±1.7) | +1.62 | > .25 | 4.09 (±0.48) | +5.96 | > .20 | 0.84 (±0.04) | -6.67 | > .05 |

[a] Results are expressed as dry weight percentages.

[b] Mean with standard deviation given in parentheses. Five determinations were made for protein and for KDO, and four were made for carbohydrate.

[c] This value represents the percent increase (+) or decrease (−) compared to the Mueller-Hinton-grown cells.

[d] Value determined by performing Student's *t* test on the means, employing the one-tailed *t* table. *P* values of <0.05 and lower were considered statistically significant.

the level of resistance to polymyxin (Table 2). For instance, cell envelopes from both the cells more resistant to polymyxin and from the cells more susceptible to polymyxin than the Mueller-Hinton-grown control cells contained slightly more total protein than the control cells. Of the three carbon sources that yielded cells more resistant to polymyxin than the control cells, the L-leucine-grown cells had a statistically significant increase in cell envelope carbohydrate content, whereas the L-glutamate-grown cells had a statistically significant decrease in cell envelope carbohydrate content compared to the control cells. Similarly, of the five carbon sources yielding cells more susceptible to polymyxin than the control cells, the cell envelope carbohydrate content was statistically significantly decreased in isobutyrate-grown cells but statistically significantly increased in L-valine-grown cells in comparison to the control cells. In general, the cell envelopes from cells grown in all the carbon sources tested had less KDO than the cell envelopes from the Mueller-Hinton-grown cells. The concentration of KDO was compared with the cellular percentage of readily extractable lipid to determine whether the fluctuations in total KDO were indicative of LPS perturbations (Table 3). None of the differences in values of the comparative ratio of KDO to readily extractable lipid was statistically significant. The stability of this ratio suggested that the backbone moiety of the core polysaccharide as measured by KDO was relatively unaffected qualitatively by these carbon sources. Furthermore, the cell envelope KDO content was not related to the level of polymyxin susceptibility. This noncorrelation was exemplified by the nearly equal concentration of KDO in isobutyrate-grown cells with an MIC of 2 U and D-glucose-grown cells with an MIC of 650 U. In summary, the cell envelopes from the cells grown on the three carbon sources yielding cells more resistant to polymyxin than the control cells did not differ significantly in their content of total protein, carbohydrate, or KDO from the cell envelopes from the cells grown on the five carbon sources yielding cells more susceptible to polymyxin.

TABLE 3. *Effects of carbon source on ratio of KDO to readily extractable lipid*

| Carbon source | KDO (mg)/REL (mg)[a] (%) |
|---|---|
| Mueller-Hinton | 0.90/9.42 (9.6) |
| D-Glucose | .65/8.10 (8.0) |
| L-Glutamate | .88/8.52 (10.3) |
| L-Isoleucine | .84/8.11 (10.4) |
| L-Leucine | .78/7.30 (10.7) |
| L-Valine | .80/8.25 (9.7) |
| DL-2-Methylbutyrate | .73/7.82 (9.7) |
| Isovalerate | .79/7.83 (10.1) |
| Isobutyrate | .69/8.10 (8.5) |

[a] Freeze-dried cells were extracted with chloroform-methanol (2:1). Readily extractable lipid (REL) was measured gravimetrically by evaporation of solvent extract.

**Polyacrylamide gel electrophoresis of the cell envelopes.** The cell envelope from the control cells grown in Mueller-Hinton broth con-

tained six protein bands in high concentration upon polyacrylamide gel electrophoresis (Fig. 1). The calculated molecular weight for each of these bands is given in Fig. 1. These proteins correspond to proteins shown previously (10) to be OM proteins. The three OM proteins of molecular weights 47,000, 36,500, and 24,000, which were greatly reduced in concentration in the polymyxin-resistant strains examined by Gilleland and Lyle (10), are denoted in Fig. 1 with asterisks. No pattern of reduction in the concentration of these three OM proteins was observed in the more resistant cells. In addition, no other pattern of protein alterations in the various cell envelopes could be correlated with the degree of susceptibility to polymyxin.

Differences in the cell envelope protein profiles were noted between the cells grown in Mueller-Hinton complex medium and cells grown in the basal media. In particular, cells grown in basal medium having D-glucose or L-leucine as the carbon source had increased concentrations of the higher-molecular-weight OM proteins (note the 47,000 and 44,000 bands in Fig. 1), whereas the Mueller-Hinton-grown cells had higher concentrations of the OM proteins of 36,500 molecular weight and lower. Differences between the protein content of the cell envelope upon growth in complex versus basal medium have been noted previously in *P. aeruginosa* (12). In addition, similar findings have been observed for another strain of *P. aeruginosa* employing different complex and basal media. Growth of *P. aeruginosa* PAO in nutrient broth yields a cell envelope with the 36,500-molecular-weight OM protein predominating, whereas growth of this strain in basal medium 2 (10) with glucose as the carbon source yields a cell envelope having greatly increased concentrations of OM proteins of 51,000 and 44,000 daltons, the appearance of a new OM band of 47,000 daltons, and a decrease in the 36,500-dalton protein band (10; Gilleland, unpublished data).

## DISCUSSION

This study was undertaken to help clarify the mechanism by which polymyxin is excluded from penetration through the OM of resistant cells. Polymyxin is a basic peptide antibiotic of approximately 1,200 molecular weight having amphiphilic properties with a charged hydrophilic headgroup and a hydrophobic side chain (13). A model has been proposed recently (13, 25) in which polymyxin interacts with membranes by the paraffin tail of the polymyxin molecule sticking into the hydrophobic part of

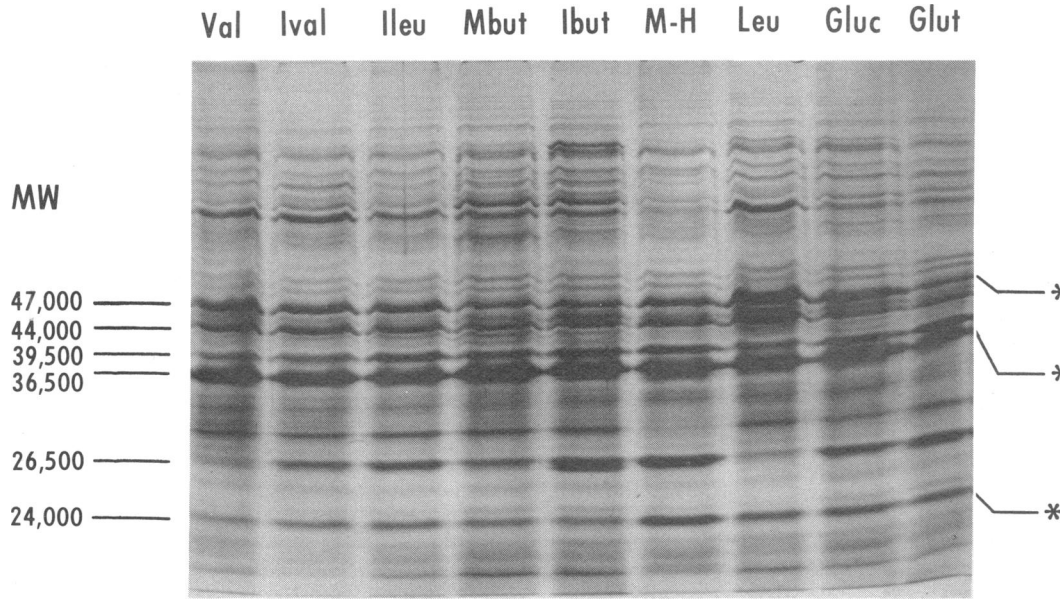


FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell envelopes of P. aeruginosa 015 grown in media with various carbon sources. The carbon sources were: Val, L-valine; Ival, isovalerate; Ileu, L-isoleucine; Mbut, DL-2-methylbutyrate; Ibut, isobutyrate; M-H, Mueller-Hinton medium; Leu, L-leucine; Gluc, glucose; and Glut, L-glutamate. The apparent molecular weights (MW) of six concentrated bands are given on the left; the three bands representing the bands that have been previously identified as OM proteins reduced in polymyxin-resistant strains are denoted by an asterisk on the right.*

the membrane while the charged ring peptide headgroup lies flat against the membrane outer surface. The insertion of the paraffin tail into the lipid bilayer results in an expansion of the lipid lattice with an asymmetric distortion or tilting of the lipid chains (13, 25), with subsequent changes in the fluidity and permeability properties of the membrane. Such a mechanism would explain the bactericidal action of polymyxin through interaction with the cytoplasmic membrane. It is far less clear how the polymyxin molecule with its large, hydrophilic headgroup penetrates through the OM of gram-negative bacteria to reach the underlying cytoplasmic membrane.

Two different basic mechanisms for polymyxin penetration through the OM of *P. aeruginosa* can be envisioned. One mechanism would be for the antibiotic molecules to interact with the lipids of the OM in the same manner as described above for the cytoplasmic membrane, leading ultimately to the transient local breakdown (19) of the OM, allowing penetration through the OM. Resistance by exclusion in this case would be determined by OM lipid alterations, perhaps through loss of receptor phospholipids, so that polymyxin no longer would bind to and interact with the OM lipid bilayer. The second penetration mechanism would be for the polymyxin molecule to initially be bound by OM LPS, phospholipids, or both, but for the actual penetration to proceed through an OM protein porin. Resistance by exclusion would be determined by loss of the appropriate OM protein that forms the porin.

There is no clear-cut evidence from the present literature to establish the correctness of either of the two possible mechanisms. There have been contradictory reports as to the presence (1, 2, 4, 6) or absence (5, 28) of lipid alterations in the cell envelopes of polymyxin-resistant cells. The absence of lipid alterations was noted in species other than *P. aeruginosa*. Furthermore, in most studies assessing lipid alterations no attempt was made to assess OM protein alterations. Gilleland and Lyle (10) recently provided evidence of the loss of OM proteins in association with the acquisition of resistance to polymyxin in *P. aeruginosa*. This would support the penetration of polymyxin through the OM via protein pores. However, their study did not assess lipid alterations in the cell envelopes of the resistant cells. It is conceivable that polymyxin resistance in a given strain might be derived from either of these suggested mechanisms or a combination thereof, depending upon the nutritional environment.

The lack of a definitive model to explain resistance despite the concerted efforts of many workers suggests that polymyxin resistance is a complex, multifaceted phenomenon. Thus, further studies which attempt to assess both the lipid and the protein alterations within the cell envelope of polymyxin-resistant cells are warranted, to establish more clearly the mechanism allowing penetration of polymyxin through the OM.

This present study examined the cell envelopes of *P. aeruginosa* 015, grown with various carbon sources which resulted in varying degrees of susceptibility to polymyxin (see Table 1), for an alteration in the OM protein and LPS content similar to that reported by Gilleland and Lyle (10) for their polymyxin-resistant strains. Although four of the six resistant strains in the Gilleland and Lyle study were resistant to >6,000 U of polymyxin per ml, their BR-6 and BR-9 isolates had levels of resistance (200 and 800 U/ml, respectively) similar to those of the 015 strain grown in L-leucine, D-glucose, or L-glutamate. These similar levels of resistance to polymyxin among certain of the isolates in the two studies made it appropriate to determine whether the cells had attained this degree of resistance through a similar loss of LPS and protein from the OM. No correlation between LPS content (Table 2 and 3) or the content of the OM proteins (Fig. 1) with polymyxin resistance was observed. Therefore, the differences in polymyxin susceptibility that resulted from growth in the various carbon sources used in this study can best be explained on the basis of the previously reported lipid alterations (4). The development of increased resistance to polymyxin in this strain under these conditions appeared to have a different physiological basis from that reported by Gilleland and Lyle, who correlated polymyxin resistance with the loss of OM protein porins.

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